Telomere length and redox balance in master endurance runners: The role of nitric oxide

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Abstract: Leukocyte telomere length (LTL), a biological marker of aging that is associated with age-related diseases, is longer in master endurance runners (ER) than age-matched controls, but the underlying mechanisms are poorly investigated. The LTL, nitric oxide (NO), and redox balance of ER master runners were analyzed and compared to untrained middle-aged and young adults. We hypothesized that NO and redox balance at baseline would be related to longer LTL in ER athletes. Participants (n = 38) were long-term ER runners (n = 10; 51.6 ± 5.2 yrs.; 28.4 ± 9.4 yrs. of experience) and untrained age-matched (n = 17; 46.6 ± 7.1 yrs) and young controls (n = 11; 21.8 ± 4.0 yrs). Volunteers were assessed for anamnesis, anthropometrics, and blood sampling. Measurements of pro-and anti-oxidant status and DNA extraction were performed using commercial kits. Relative LTL was determined with qPCR analyses (T/S). While the middle-aged controls had shorter LTL than the young group, no difference was observed between ER athletes and young participants. A large effect size between the LTL of ER athletes and middle-aged controls (d = 0.85) was also observed. The ER athletes and untrained young group had better redox balance according to antioxidant/pro-oxidant ratios compared to middle-aged untrained participants, which also had lower values for redox parameters (TEAC/TBARS, SOD/TBARS, and CAT/TBARS; all $p < 0.05$). Furthermore, the NO level of ER athletes (175.2 ± 31.9 μM) was higher ($p < 0.05$) than middle-aged controls (67.2 ± 23.3 μM) and young participants (129.2 ± 17.3 μM), with a significant correlation with LTL ($r = 0.766$; $p = 0.02$). In conclusion, ER runners have longer LTL than age-matched controls, which in turn may be related to better NO bioavailability and redox balance status.

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ORIGINAL INVESTIGATION

TELOMERE LENGTH AND REDOX BALANCE IN MASTER ENDURANCE RUNNERS: THE ROLE OF NITRIC OXIDE

Running head: The role of nitric oxide on telomere biology

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ABSTRACT

Leukocyte telomere length (LTL), a biological marker of aging that is associated with age-related diseases, is longer in master endurance runners (ER) than age-matched controls, but the underlying mechanisms are poorly investigated. The LTL, nitric oxide (NO), and redox balance of ER master runners were analyzed and compared to untrained middle-aged and young adults. We hypothesized that NO and redox balance at baseline would be related to longer LTL in ER athletes. Participants (n=38) were long-term ER runners (n=10; 51.6±5.2yrs; 28.4±9.4yrs of experience) and untrained age-matched (n=17; 46.6±7.1yrs) and young controls (n=11; 21.8±4.0yrs). Volunteers were assessed for anamnesis, anthropometrics, and blood sampling. Pro-oxidants, antioxidants, and DNA extraction were measured using commercial kits. Relative LTL was determined with qPCR analyses (T/S). While the middle-aged controls had shorter LTL than the young group, no difference was observed between ER athletes and young participants. A large effect size between the LTL of ER athletes and middle-aged controls (d=0.85) was also observed. The ER athletes and untrained young group had better redox balance according to antioxidant/pro-oxidant ratios compared to middle-aged untrained participants, which also had lower values for redox parameters (TEAC/TBARS, SOD/TBARS, and CAT/TBARS; all p<0.05). Furthermore, the NO level of ER athletes (175.2±31.9µM) was higher (p<0.05) than middle-aged controls (67.2±23.3µM) and young participants (129.2±17.3µM), with a significant correlation with LTL (r=0.766; p=0.02). In conclusion, ER runners have longer LTL than age-matched controls, which in turn may be related to better NO bioavailability and redox balance status.

Keywords: aging; performance; oxidative stress; running; master athlete; longevity

INTRODUCTION

Leukocyte telomere length (LTL) is a well-known marker of biological age, being negatively associated with a risk of developing cardiovascular and metabolic diseases (Blackburn et al. 2015). A healthy lifestyle seems to reduce telomere attrition and attenuate cellular aging, improving general health and the lifespan (Denham et al. 2016a).

Exercise training is shown to prevent disease through telomere length maintenance, but little is known about the optimal amount or exercise mode that leads to healthier outcomes (Denham et al. 2016a). Long-term endurance training (ER) has been shown to attenuate telomere shortening (Borghini et al. 2015; Denham et al. 2016b; LaRocca et al. 2010; Osthus et al. 2012), although this is not unequivocal (Rae et al. 2010). Moreover, LTL is positively associated with maximal oxygen uptake (VO_2max) (Borghini et al. 2015; Denham et al. 2016a; Denham et al. 2016b; LaRocca et al. 2010; Osthus et al. 2012; Rae et al. 2010), but the possible mechanisms by which chronic exercise leads to longer LTL need to be more thoroughly studied. Denham et al. (2016b) investigated LTL and telomere-regulating genes in ER adults and reported that besides longer LTL, they also demonstrated upregulated TERT and TPP1 in comparison to healthy controls. Likewise, other biological markers, e.g., nitric oxide (NO) and redox balance, can be directly and indirectly associated with the modulation of telomere length in ER athletes.

Previous studies have reported that ER elicits greater NO availability(Xie et al. 2012). NO has beneficial effects on bioenergetic homeostasis, including stimulation of fatty acid and glucose oxidation (Dai et al. 2013). Furthermore, NO plays an important

role in redox signaling in both physiological and pathological conditions(Pacher et al. 2007).

NO is known to induce vasodilation, regulate blood flow, improve glucose uptake, and elicit mitochondrial biogenesis (Stamler and Meissner 2001), and therefore it is commonly associated with endurance performance (Bailey et al. 2009). However, acute endurance exercise also triggers modifications in redox homeostasis including superoxide (O_2^-) production via the activated respiratory burst of phagocytic cells (NADPH) and peroxynitrite ($ONOO^-$) formation through reactions with O_2 (Powers et al. 2011). Therefore, the imbalance between pro-oxidant production and antioxidant defenses may lead to DNA damage, increasing telomere attrition and, in turn, premature cellular aging, favoring the development of chronic diseases (Blackburn et al. 2015).

In that regard, some studies have shown the possible association between NO activity and cellular aging (Fernandez-Moreno et al. 2011; Matsui-Hirai et al. 2011), in which reduced levels of NO would decrease the scavenger of O_2^- (or lead to an antioxidant inability to neutralize it), resulting in increased production of $ONOO^-$. Therefore, the outcome of reduced NO availability is increased pro-oxidant potential, which in turn would increase telomere attrition (Fernandez-Moreno et al. 2011; Matsui-Hirai et al. 2011).

It is noteworthy that physical exercise training of any kind seems to improve the production of antioxidant indicators and reduces pro-oxidants (Sousa et al. 2017), in addition to increasing the production of endothelium-derived NO (Green et al. 2004). Nevertheless, the possible association of NO with telomere length has not yet been tested in long-term ER adults. Therefore, young endurance athletes have longer LTL and more NO, but after 20 years of continuous practice the scenario is the same?

In light of that, we aimed to investigate LTL, redox balance, and NO in long-term middle-aged ER runners and untrained controls (age-matched and young). We hypothesized that master ER athletes would have longer LTL, better redox balance, and higher NO levels than untrained age-matched controls and that redox balance and NO would be positively associated with LTL.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Human Research Ethics Committee of the Catholic University of Brasília (protocol number: 1.201.316) and was conducted according to the Declaration of Helsinki. Furthermore, all subjects provided written informed consent before participation after all procedures had been clearly explained.

Subjects

Master athletes were recruited from local races and personal recommendations from athlete to athlete. Inclusion criteria for all participants were: no history of inflammatory or metabolic diseases or cancer; non-smokers; and no regular use of drugs, including hormone replacement therapies. Long-term ER athletes criteria included: men aged between 40 and 70 years old and at least 15 years of regular and competitive practice in endurance races (10km to marathon). Age-matched and younger controls had to be untrained to participate in the study.

We aimed to evaluate ER athletes with exceptional experience. Thus, our master athletes had 28.4 ± 9.4 years of training and averaged 7.1 ± 4.1 competitions per year.

The maximal time of uninterrupted inactivity (e.g., due to injury, personal reasons, etc.) was 10.7 ± 17.1 months. The final sample size (n = 38) was comprised of 10 ER men, 17 middle-aged untrained men, and 11 young untrained adults. The characteristics of the sample are displayed in Table 1. For the main analyses, the total sample size conferred a statistical power of 90% considering the significance level at 5% ($\alpha = 0.05$) and moderate effect size ($d = 0.6$).

Methodology

All volunteers arrived at the laboratory in an 8-hour fasting state for blood sample collection (two samples of ~4mL each using Vacutainer tubes with EDTA), anamnesis, and anthropometric measures. Relative body fat was estimated using the seven-fold protocol proposed by Jackson and Pollock (1978). A single researcher measured all skinfolds with the Lange® caliper (Cambridge Scientific Instruments, Maryland, USA). Blood samples were centrifuged for plasma isolation and biochemical analysis of NO and parameters of redox balance. Mononuclear cells were isolated from blood, as described elsewhere (Boyum 1968).

Plasma samples were analyzed to determine NO, superoxide dismutase activity (SOD), catalase activity (CAT), total antioxidant capacity by the trolox-equivalent antioxidant capacity (TEAC), and lipid peroxidation by the thiobarbituric acid-reactive substances (TBARS).

Nitric Oxide

NO was measured using a Griess reaction, according to the following protocol. Plasma samples were deproteinized with zinc sulfate (20%), 100μL of each sample was

dispensed in duplicate in a 96-well plate, 100µL of vanadiumchloride, 50µLsulfinylamide, and 50µL of *N*-(1-Naphthyl)ethylenediaminedihydrochloride were added to the samples, and standard nitrite curves were prepared. After the addition of the aforementioned reagents, the plates were homogenized and incubated for 40 minutes at 37°C. The spectrophotometer reading was made at 540nm.

Lipid Peroxidation

Assessment of TBARS is one the most used methods to determine lipid peroxidation and oxidative damage in cells and tissues. The protocol used in the present study was adapted from Ohkawa et al. (1979).

Serum samples were diluted in 320µL MiliQ H_2O (1:5), and then 1mL of trichloroacetic acid (TCA) 17.5%, pH 2.0 and 1mL of thiobarbituric acid (TBA) 0.6%, pH 2.0 were added, respectively. After homogenization, the samples were kept in a water bath for 30 minutes at 95°C. The reaction was interrupted with the immersion of the microtubes in ice and the addition of 1mL of TCA 70%, pH 2.0 and another incubation for 20 minutes at room temperature.

After centrifugation (3,000rpm for 15 minutes), the supernatant was removed and put in new microtubes and read by spectrophotometry at 540nm. The concentration of lipid peroxidation products was calculated using the molar extinction coefficient equivalent for malondialdehyde (MDA-equivalent = $1.56x10^5$ $M^{-1}cm^{-1}$).

Antioxidant Parameters

The three antioxidant parameters used in this study were measured using commercial kits and following the manufacturers' protocols. SOD activity was

measured using an assay kit (Sigma Aldrich®, California, USA) with a final spectrophotometric reading at 450nm. Total antioxidant capacity was measured with a trolox-equivalent assay kit (QuantiChrom BioAssay Systems, California, USA). Catalase activity was measured using the Amplex™ Red assay kit (Thermofisher Scientific®, USA) with a final spectrophotometric reading with one minute incubation at 560nm.

DNA Extraction and Telomere Length

DNA was extracted from peripheral blood mononuclear cells (PBMC) performed with the PureLink® Genomic DNA minikit (Life Technologies®), according to the manufacturer's instructions. Quality and quantity of each DNA sample were analyzed by spectrophotometry using a NanoDrop (ThermoFisher, Wilmington, USA) with a ratio of absorbance at 260/280 nm, considering a range between 1.7 and 2.0 of purity.

PCRq reactions utilized 20ng (4µL of 5ng/µL) DNA solution, 100nM (1µL) of each primer (direct and reverse), 10µL of SYBR Green (Fast SYBR Green Master Mix Thermo Scientific), and 4µL of Milli-Q H_2O in final volume of 20µL. The reactions were performed in triplicate for each sample on separate plates. On each plate, a negative control amplification reaction (water) and reference sample amplification were performed in triplicate, from the serial 1:2 dilution, to determine the efficiency of the amplification reaction with each of the primer pairs (telomeric sequence and single copy gene sequence - T/S). This efficiency curve consisted of six points. The largest amount of DNA in the reference sample was 40ng and the lowest was 1.25ng (serial 1:2 dilution from 40ng). For the amplification efficiency of the primers (telomeric sequence and

36B4) of the reference sample, it was serially diluted 1:2 from 40ng to 1.25ng (six points). The efficiency of the amplification was determined by the equation $E = (10^{-1/slope^{-1}}) \times 100$.

Statistical Analysis

The normality and homogeneity of the data were tested by the Shapiro-Wilk and Levene's tests, respectively. Data were expressed as mean ± standard deviation. To compare all outcome variables between groups, one-way analyses of variance with Bonferroni corrections to minimize type 1 errors were utilized. A secondary analysis was performed stratifying the LTL data by tertiles, in which subjects with shorter and longer LTL had their redox state compared with Student's *t*-tests for independent variables. Furthermore, Cohen's *d* was used to verify the effect size of LTL comparisons (Cohen 2013). Associations between outcome variables were performed using Pearson's product-moment correlations. The significance level for all tests was set at $p < 0.05$. All analyses were performed with SPSS 21 (IBM, Inc., Illinois, USA), G*Power (v3.1) and GraphPad Prism (v6.0).

RESULTS

While the ER athletes and untrained peers were similar in age, the younger untrained group differed in comparison to the other two groups. The untrained middle-aged group also had a higher BMI *than the young group and ER* and a higher percent body fat than the young group (Table 1).

*** Table 1 ***

The ER group had a lower total antioxidant capacity compared to the younger adults. Untrained middle-age adults had lower SOD and CAT activity compared to younger adults and lower CAT activity compared to ER athletes (Table 2).

***** Table 2 *****

The ER athletes and young adults had better redox balance according to antioxidant/pro-oxidant ratios compared to untrained middle-age adults. The younger adults had higher values of TEAC/TBARS than the other two groups (Figure 1-A). SOD/TBARS and CAT/TBARS ratios were lower in the middle-age untrained group (Figures 1-B and 1-C). Furthermore, the ER group had higher values of NO than the other two groups (Figure 1-D).

Additionally, the untrained age-matched group had shorter LTL than younger adults, with no statistical difference between ER athletes and young adults (Figure 1-E). A large effect size was found between the ER group and both the untrained aged-matched adults (d = 0.85) and the younger group (d = 0.80) according to the Cohen's qualitative standard (Cohen 2013). The untrained age-matched group had higher oxidative stress according to the redox index ([CAT+SOD/TBARS]) in comparison to the other groups (Figure 1-F).

***** Figure 1 *****

A significant correlation was found (large effect) between LTL and NO for the ER group only (Figure 2-C). No significant correlations between LTL and NO were identified for the other groups or when grouping all subjects. Chronological age was negatively associated with LTL when analyzing all subjects (r = -0.39; p = 0.02).

***** Figure 2 *****

When participants were stratified in tertiles according to those with longer and shorter LTL, individuals with longer LTL showed higher CAT activity and elevated CAT/TBARS ratio and NO (p< 0.05). Moderate effects were noted for SOD activity, SOD/CAT ratio, and redox index in favor of longer LTL (Figures 3). The tertiles groups were composed as follows: shorter LTL (2 young; 8 untrained; 1 ER); longer LTL (6 young; 1 untrained; 5 ER); the other subjects were allocated as intermediary LTL.

***** Figures 3 and 4 *****

DISCUSSION

The aim of this study was to analyze LTL, redox balance, and NO in middle-aged long-term ER athletes and untrained controls (age-matched and young). The main results indicated that long-term ER athletes have greater NO availability and better redox balance, possibly attenuating telomere attrition.

The reaction of NO with O_2^-makes the formation of $ONOO^-$ inevitable *in vivo*, which is a powerful reactive nitrogen species (RNS) (Forstermann et al. 2017; Pacher et

al. 2007). Therefore, the overproduction of or inability to neutralize O_2^- possibly leads to increased oxidative stress and lowers the availability of NO. The main mechanism to scavenge O_2^- is by SOD activity forming reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) (Forstermann et al. 2017; Kar et al. 2012). Therefore, elevated SOD activity can indicate greater antioxidant capacity, but also induces the formation of ROS. Regarding the present results, in which untrained adults had lower levels of NO, SOD activity, and SOD/TBARS ratio, lower SOD activity possibly leads to an overproduction of $ONOO^-$ by O_2^- scavenging NO, thus increasing oxidative stress.

Downstream from the antioxidant complex to neutralize ROS is the reaction of H_2O_2 with CAT forming H_2O and O_2 (Forstermann et al. 2017; Pacher et al. 2007). Thus, the elevated activity of CAT in young untrained adults and ER athletes may be enough to neutralize ROS arising from mitochondrial distress, NADPH oxidase, and xanthine oxidases (Bedard and Krause 2007; Wang et al. 2014). The increased CAT activity reduces NOS inhibition, whereas the elevation of H_2O_2 increases the inhibition of such enzyme (Brennan et al. 2002). Therefore, the increased CAT activity possibly leads to reduced H_2O_2 and enhanced eNOS activity increasing NO availability, as observed in the ER athletes in the present study.

The reduced antioxidant activity and/or overproduction of ROS and RNS are possibly the main factors contributing to increased telomere attrition, since telomeres protect the genome from DNA damage by attracting oxidative damage to noncoding telomere sequences (Kawanishi et al. 1999). The possible relationship between NO and telomere length has been shown by others (Compton et al. 2006; Farsetti et al. 2009; Fernandez-Moreno et al. 2011; Matsui-Hirai et al. 2011; Vasa et al. 2000). Compton et al. (2006) found that by inhibiting 90 kDa heat-shock protein (Hsp90), which

functionally works with NOS by converting L-arginine to L-citrulline and NO, significant increases in free radical production and telomere damage were demonstrated. Furthermore, evidence exists of a positive relationship between NO availability and telomerase activity, a ribonucleo-protein that synthesizes the telomeric repeats at the end of chromosomes (Farsetti et al. 2009; Vasa et al. 2000).

Our findings of increased NO availability correlated with longer LTL for master ER athletes might be explained by two possible pathways: (a) scavenging free radicals and diminishing telomere attrition, and (b) increasing telomerase activity and consequently telomere maintenance. The present investigation did not measure telomerase activity or the expression of sheltering complex, and the sample size was low (especially for athletes), what in turn may be considered as limitations. However, although preliminary, to the best of our knowledge this is the first study to analyze the relationship between increased NO availability and LTL in long-term ER men, and a possible role of the redox state. The non-association of NO with LTL in non-athletes still needs elucidation. Possibly the increase in NO to scavenge free radicals is not the primary pathway to reduce oxidative stress, since a reaction of NO with O_2^- generates another powerful pro-oxidant, peroxynitrite ($ONOO^-$) (Radi 2018), that without proper antioxidant defenses can lead to a premature cell aging. On the other hand, for the athletes of present study who have higher levels of both NO and longer LTL, the NO may have had a role on the increased anti-oxidant defenses and thus attenuation of telomere attrition. In conclusion, lifelong endurance training seems to improve both NO availability and redox balance, contributing to lower telomere attrition and longer LTL in master athletes compared to untrained age-matched controls. Participants with longer LTL had increased CAT and elevated NO availability, which seem to be key

elements in protecting telomeres. Further research should focus on different groups of master athletes (i.e., sprinters vs. endurance runners) and the possible relationships between redox state, telomerase activity, and sheltering proteins in response to different lifetime training methods. Reduced levels of NO and elevated oxidative stress may indicate early cellular senescence, favoring the development of age-related diseases.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Redox balance, nitric oxide (NO), and leucocyte telomere length (LTL) of young, middle-aged untrained, and master endurance-trained athletes. TBARS: thiobarbituric acid-reactive substances; TEAC: trolox-equivalent antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; one-way ANOVA with Bonferroni *post hoc*; *: different from the young group; #: different from the endurance group; *d*: effect size (Cohen's *d*). Data expressed as mean ± standard deviation.

Figure 2. Linear regression and Pearson's product-moment correlation between leucocyte telomere length (LTL) and nitric oxide (NO) of young (A), middle-aged untrained (B), and master endurance-trained athletes (C).

Figure 3. Redox balance comparison between participants with longer and shorter leucocyte telomere length (LTL). *: statistical difference (t-test for unpaired samples); ef: effect size (Cohen's *d*). Data expressed as mean ± standard deviation.

Figure 4. Schematic representation of the possible physiological mechanisms linking nitric oxide levels to telomere dynamics. Straight line: represents the possible pathway of long-term endurance athletes with increased NO availability, redox balance, and longer telomere length. Dashed line: represents the possible pathway of untrained middle-aged controls, with low NO availability, higher oxidative stress, and shorter telomeres.

Table 1. Age and body composition of young, middle-aged untrained controls, and master endurance-trained athletes. Data expressed as mean ± standard deviation.

	Young (n = 11)	Untrained (n = 17)	Master Athletes (n = 10)
Age (years)	21.8 ± 4.0	46.6 ± 7.1*	51.6 ± 5.2*
BMI ($kg \cdot m^{-2}$)	23.8 ± 2.5	31.2 ± 6.9*†	22.8 ± 1.9
Body fat (%)	9.8 ± 3.6	24.4± 5.0*†	12.7 ± 4.4

BMI: body mass index; *: $p<0.05$ in relation to the young group; †: $p<0.05$ in relation to the Master endurance-trained group.

Table 2. Oxidative stress variables of young controls, untrained age-matched controls, and master endurance-trained athletes. Data expressed as mean ± standard deviation.

	Young (n = 11)	Untrained (n = 17)	Master Athletes (n = 10)
TBARS ($nmol \cdot L^{-1}$)	9.6 ± 1.2	10.8 ± 1.8	9.8 ± 2.3
TEAC (μM)	1016.1 ± 194.9	841.1 ± 148.8	739.1 ± 206.5*
SOD ($U \cdot mL^{-1}$)	80.3 ± 14.1	55.0 ± 23.1*	71.8 ± 23.5
CAT ($U \cdot \mu L^{-1}$)	822.1 ± 175.3	460.1 ± 265.5*†	752.6 ± 270.9

TBARS: thiobarbituric acid-reactive substances; TEAC: trolox-equivalent antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; *: $p<0.05$ in relation to the young group; †: $p<0.05$ in relation to the master endurance-trained group.